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REVIEW

Cardiac regeneration in vivo: Mending the heart from within?

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Abstract A growing body of evidence has shown that the heart is not terminally differentiated but continues to renew its cardiomyocytes even after the neonatal period. This new view of the heart increases hope for changing the strategy for treating cardiac injuries toward regenerative approaches. However, the magnitude and clinical significance of this process in homeostasis and disease and the underlying cellular and molecular mechanisms have been heavily debated. Numerous candidates for so-called cardiac stem cells (CSCs) have been proposed, but the different characteristics of these candidates make it difficult to identify the inherent source of regeneration. In this review, we revisit the field of cardiac stem cells and endogenous regeneration to elaborate how these fields may contribute to future regenerative strategies.

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Introduction

Most studies agree that the adult heart continues to renew cardiomyocytes even after the neonatal period. Cardiomyocytes can be generated by self-duplication and

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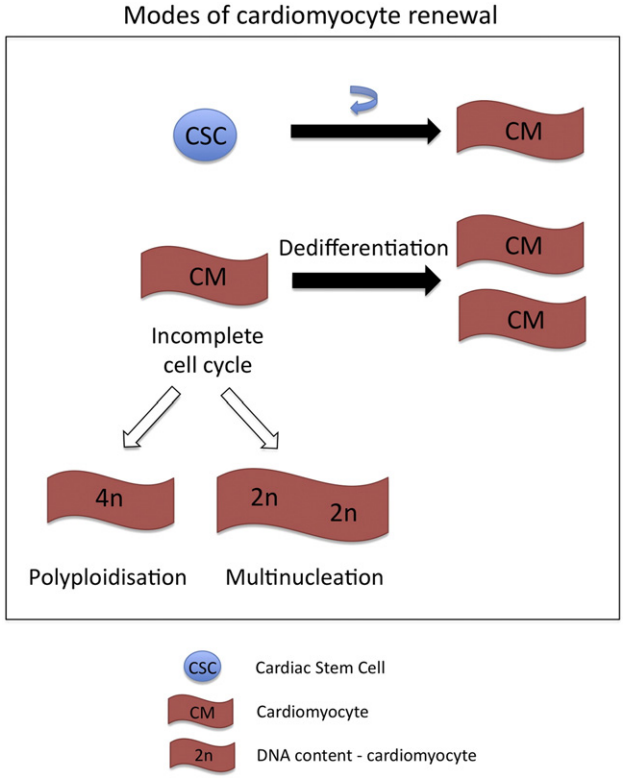

Figure 1 Modes of cardiomyocyte renewal. Adult-born cardiomyocyte can be derived from a CSC pool or by self-replication, possibly involving dedifferentiation. Both modes have been documented and might exist in parallel. A fraction of cardiomyocytes entering the cell cycle exit prematurely and becomes polyploid and/or multinucleated.

by cardiac stem cells (CSCs) (Fig. 1). Both modes of cardiomyocyte renewal have been proposed at different ages and after cardiac injury. However, the magnitude of myocyte turnover in homeostasis and disease has been heavily debated. In this review, we will provide an overview of cardiomyocyte renewal, with a focus on human hearts, and reveal potential pitfalls and misinterpretations. Furthermore, we will elaborate how endogenous repair mechanisms can be exploited for future regenerative strategies.

Magnitude of adult cardiomyocyte turnover

Evidence shows that the mammalian heart retains the capability to renew cardiomyocytes during adulthood. However, the magnitude of myocyte renewal in adult mammals, particularly in humans, is controversial (see Table 1 for adult mouse and human turnover rates). We and others have independently reported that the adult human heart has low but detectable regenerative capacity (Bergmann et al., 2009; Mollova et al., 2013), whereas other groups, mainly one research group, have reported that the human heart has the regenerative capacity to renew completely within 5 years or even more rapidly after cardiac infarction (Kajstura et al., 1998, 2010a,b).

One argument for the markedly high turnover of myocytes has been the detection of apoptotic and necrotic myocytes (Anversa et al., 2013). Indeed, cell death has been found in cardiac pathologies and in healthy myocardium (Mallat et al., 2001; Olivetti et al., 1997; Saraste et al., 1999). The critical parameters to establish the magnitude of cell death are the frequency of dying cells and the duration of an apoptotic-necrotic cell phenotype. To date, there is no consensus on the length of the apoptotic-necrotic cell phenotype in cardiomyocytes. Estimates range from a few hours to days, making the extrapolation of death rates per year or even over a lifetime problematic (De Saint-Hubert et al., 2009; Rodriguez and Schaper, 2005; Takemura et al., 2013). Moreover, the TUNEL technique, which detects apoptosis by identifying DNA nicks, is not solely specific for programmed cell death and might also label cells undergoing DNA repair (Kanoh et al., 1999).

Table 1 Cardiomyocyte renewal in adult hearts. Cardiomyocyte: CM; immunohistochemistry: IHC; NR: not reported; phospho-histone H3: p-H3; *converted to percentage per year.

Study	Species	Adult CM renewal per year (%)	Renewal after injury/in diseased hearts	Methodology
Bergmann et al., 2009	Human	1% to less than 0.5%	NR	^{14}C dating
Kajstura et al., 1998	Human	10.5%*	Increased	Mitotic index
Kajstura et al., 2010a	Human	7%–40%	NR	IHC (apoptosis, proliferation, senescence)
Kajstura et al., 2010b	Human	7.3%–51.1%*	NR	IdU labeling (cancer patients)
Mollova et al., 2013	Human	1.6% to 0.04%	NR	Mitotic index (p-H3)
Bersell et al., 2009	Mouse	No renewal [20,501 CMs analyzed (p-H3)]	Increased	Mitotic index (p-H3) and aurora B labeling
Hosoda et al., 2009	Mouse	50%–80%*	Increased	BrdU labeling
Malliaras et al., 2013	Mouse	1.3%–4.0%	Increased	BrdU labeling
Senyo et al., 2013	Mouse	0.76%	Increased	^{15}N-thymidine labeling
Soonpaa and Field, 1997	Mouse	<1%*	Increased	^{3}H-thymidine labeling
Walsh et al., 2010 and personal communication	Mouse	No renewal (300,000 CM nuclei analyzed)	NR	BrdU labeling

Historically, cardiomyocyte renewal in humans has been difficult to establish. The most common tools to study proliferation have been immunohistochemical markers of proliferation such as Ki67 or the mitotic marker phosphohistone 3 (p-H3). However, because only a short glimpse of the proliferating cells can be achieved, establishing turnover dynamics in cell populations is problematic. For example, whether only a small subpopulation of cells renews and the majority of cells are quiescent remains unknown. This scenario would cause a dramatic overestimation of the overall cell turnover. Another shortcoming of this strategy is that cardiomyocytes derived from a stem cell population would not be detected or the number would be underestimated because only cycling myocytes that already expressed a myocyte lineage commitment would be identified. A birth marker that is incorporated into CSCs or duplicating cardiomyocytes is required to chase newborn myocytes and establish their number and survival in the human heart. Thymidine analogs such as bromodeoxyuridine (BrdU) or iododeoxyuridine (IdU), which are incorporated in the S-phase of the cell cycle and can be detected by antibody labeling, are powerful tools that have helped establish the lineage of stem cell populations in various organ systems, including the brain. In a seminal study by Eriksson et al., brain specimens from cancer patients who received BrdU at a diagnostic dosage were analyzed for BrdU incorporation in adult neurons in the dentate gyrus of the hippocampus (Eriksson et al., 1998). Importantly, these patients had not received any cancer treatment prior to or at the time of the BrdU infusion. Using the same strategy, Kajstura et al. reported a high number of IdU-positive cardiomyocytes (2.5%–46%) in cancer patients who received IdU mostly in therapeutic dosages (approximately 10-fold higher than the diagnostic IdU dosage) along with radiotherapy directed to different organs (Kajstura et al., 2010b). The high frequency of IdU-positive cardiomyocytes was similar to fibroblasts, even exceeding the labeling frequency of endothelial cells, and (in some tumor tissues) raises questions regarding the interpretation of the data (Laflamme and Murry, 2011). Moreover, given the reported high renewal rate of cardiomyocytes, the IdU label would be diluted out within the first 5 cell divisions shortly after the last IdU pulse (Wilson et al., 2008). Even including the possibility of label retention in dividing cardiomyocyte and stem cells, labeling frequencies of 24% and 46% are incompatible with extensive chasing periods of 1472 and 660 days, respectively, until the death of the patients (see supplement Bergmann et al., 2011). Thus, the fraction of IdU-labeled cardiomyocytes cannot be explained only by renewal, and other possibilities (such as aberrant DNA synthesis) must be considered (Burns et al., 2007). The reported 64-fold higher apoptosis rate of myocytes in the IdU study compared with healthy controls also questions the health status of the examined hearts, which might partly explain the high frequency of IdU incorporation (Kajstura et al., 2010b; Olivetti et al., 1997). Human myocytes show extensive polyploidy during growth and in disease (as discussed below). This process complicates the use of IdU labeling when ploidy levels are not taken into consideration (Bergmann et al., 2011).

^{14}C retrospective dating is a new technology to overcome limitations in measuring cardiomyocyte renewal in human hearts (Bergmann et al., 2009, 2012; Huttner et al., 2014; Spalding et al., 2013). This strategy is based on the incorporation of nuclear test bomb-derived ^{14}C into genomic DNA; therefore, it provides a cumulative measure for cellular turnover that is different from immunohistochemical strategies (Spalding et al., 2005).

To determine the myocardial turnover, the correct identification of cardiomyocytes and/or cardiomyocyte nuclei is critical and has been challenging (Ang et al., 2010). Because most archived heart tissue is only available frozen, an isolation strategy based on the cellular level is not feasible. Rather, we identified three independent markers [cardiac troponins I and T and pericentriolar protein 1 (PCM-1)] to identify and isolate cardiomyocyte nuclei (Bergmann et al., 2009, 2011; Bergmann and Jovinge, 2012). PCM-1, a protein associated with the centrosome complex, accumulates perinuclearly in a cell type-specific fashion in mature cardiomyocyte and skeletal muscle cells (Fant et al., 2009; Srsen et al., 2009). PCM-1 labeling identifies cardiomyocyte nuclei for isolation in different species, including mice and humans, in fresh and frozen post-mortem tissues (Bergmann and Jovinge, 2012; Bergmann et al., 2011). The insoluble perinuclear matrix remains in most phases of the cell cycle but disassembles only in pro-metaphase and metaphase of mitosis (Srsen et al., 2009), making it possible to visualize myocyte nuclei almost throughout the whole cell cycle.

Applying the ^{14}C birth dating strategy to human cardiomyocytes in the left ventricle, we have reported a low but detectable annual turnover rate that declines to values below 0.5% in aged subjects (Bergmann et al., 2009). Our measured ^{14}C concentrations are not compatible with a massive increase in the number of cardiomyocytes in growing hearts, as suggested in rodents (1.4-fold) and in young humans (3.4-fold) (Mollova et al., 2013; Naqvi et al., 2014). The increase in cardiomyocyte count in preadolescent rodents, however, might correspond to the increase in ploidy in humans, as discussed below.

Ploidy and Multinucleation of Cardiomyocytes

When cardiomyocytes enter the cell cycle, they do not always progress through the entire cell cycle and generate new daughter cells (Fig. 1). Instead, during physiological heart growth, a large fraction (studies report from 12% to values exceeding 50%) of human cardiomyocytes undergo polyploidization mainly during the physiological growth of the heart (Adler, 1991; Bergmann et al., 2009; Mollova et al., 2013; Takamatsu et al., 1983). During adulthood, the DNA content per nucleus remains constant in healthy hearts throughout life (Bergmann et al., 2009). In contrast, in murine cardiomyocytes, where only a small fraction of cardiomyocyte nuclei become polyploid (Adler et al., 1996; Bergmann and Jovinge, 2012), 93%–95% of all myocytes have become binucleated by 2 weeks after birth (Soonpaa et al., 1996; Walsh et al., 2010), and there is also a small fraction of tri- and tetranucleated cardiomyocytes at 3 months (Rota et al., 2007). Analysis of the gene expression patterns shows a down-regulation of genes involved in cell cleavage and cytokinesis at the beginning of binucleation (Walsh et al., 2010). One might speculate that binucleation is caused mainly by halted cytokinesis rather than fusion. Moreover, not only physiological heart growth but also various heart diseases, including congenital heart disease (Adler, 1976), adverse remodeling after cardiac infarction (Herget et al.,

1997; Meckert et al., 2005) and cardiac hypertrophy are triggers for extensive polyploidization (Vliegen et al., 1995). Interestingly, patients with end-stage dilated cardiomyopathy treated with a left ventricular assist device (LVAD) as a bridge for heart transplantation show a decreased ploidy level, indicating either the selective death of larger cardiomyocytes with high ploidy levels or the new formation of small cardiomyocytes with diploid nuclei (Rivello et al., 2001; Wohlschlaeger et al., 2010).

Apart from genetic fate-mapping strategies, both Ki-67 labeling and birth dating with thymidine analogs or ^{14}C might be confounded by polyploidization and multinucleation. Analyses should therefore be performed at the nuclear level. Thus, additional measures are required to establish turnover in cardiomyocytes. In contrast, the incorporation of thymidine analogs or ^{14}C into mononucleated and diploid cardiomyocytes always marks an adult-born cardiomyocyte and cannot be attributed to cell or nuclear ploidy. Cytokinesis with the formation of the cleavage furrow is the last step completing cellular division. Aurora B kinase is a chromosomal passenger protein that is localized in different subcellular compartments during mitosis. During cytokinesis, aurora B kinase targets required proteins of the contractile ring at the site of cytoplasmic separation. Detecting aurora B kinase by immunohistochemistry at the cleavage furrow is a method for visualizing cytokinesis and thereby demonstrating cell division (Tatsuka et al., 1998). A stringent analysis of myocyte renewal that accounts for the above-described confounders is crucial for establishing the magnitude of myocyte proliferation, particularly when studying diseased hearts. Because ^{14}C birth dating can account for polyploidization either by selective ^{14}C dating of diploid cardiomyocytes or by mathematical correction for measured ploidy, it is also possible to investigate renewal in heart diseases, in which the ploidy levels are higher than in healthy hearts (Bergmann et al., 2009).

CSCs as a source of adult cardiomyocytes

One decade has passed since Beltrami and co-workers provided evidence for the existence of c-kit-expressing cardiac stem cells (CSCs) (Beltrami et al., 2003). The presence of cells that express c-kit, the stem cell factor receptor, in the absence of any hematopoietic lineage markers, may allow for the generation of all major lineages in the heart, including cardiomyocytes, endothelial cells and mesenchymal cells in vitro and in vivo (Beltrami et al., 2003). Several other putative CSCs in the adult heart have been reported since then [for a review, see (Stamm et al., 2009)]. CSCs typically express adult stem cell genes, such as c-kit, stem-cell antigen 1 (Sca-1) (Oh et al., 2003) or multidrug resistance protein 1 (MDR1). Other CSCs have been identified as side population (SP) cells (Martin et al., 2004), perivascular cells (Galvez et al., 2008) or cardiosphere-derived cells (Smith et al., 2007). Although studies have reported their cardiogenic potential in vitro and in various transplantation paradigms (Passier et al., 2008), appropriate in vivo fate-mapping strategies for most stem cell markers have been lacking until recently. However, Islet1 (Isl1) stem cells were described by lineage tracing during development (Moretti et al., 2006). Isl1 cells can be isolated from the neonatal heart and then expanded and differentiated into cardiomyocyte in vitro (Laugwitz et al., 2005). Because the number of Isl1 cells decreases soon after birth, these cells resemble a remnant progenitor population from development. A potential new CSC population in the epicardial layer of the heart has been reported. Adult lineage-tracing revealed a re-activation of Wilms tumor 1 (Wt1)-expressing epicardial cells under ischemic conditions, and the ligand thymosin beta-4 could further increase their number. However, the total contribution of these cells to new myocytes is limited (Smart et al., 2011).

The cardiomyogenic potential of c-kit-expressing cells is likely the most studied aspect among the described CSCs. The proliferation and survival of hematopoietic stem cells, germ cells and other lineages is dependent on c-kit activation by binding to its ligand the stem cell factor (SCF). The role of residing c-kit-expressing cells in the postnatal and adult heart is, however, much more controversial. Using a BAC transgenic mouse that expresses EGFP under transcriptional control of the kit locus, Tallini and colleagues showed that clonally isolated and differentiated EGFP (c-kit)-positive cells from the neonatal mouse heart can give rise to cardiomyocytes, smooth muscle cells, and endothelial cells (Tallini et al., 2009). Zaruba et al. obtained a similar result when isolating c-kit-positive cells by flow cytometry from a transgenic myocyte reporter mouse (Zaruba et al., 2010). In response to cryo- and ischemic injury, EGFP (c-kit) expression is not limited to undifferentiated cells but is also found in three cardiac lineages, indicating that multipotent progenitor cells express c-kit at the neonatal stage (Jesty et al., 2012; Tallini et al., 2009). In contrast, in adult hearts, the number of c-kit-expressing cells decreases dramatically, and EGFP (c-kit) expression is restricted to endothelial cells and smooth muscle cells, suggesting that they have a role as vascular progenitor cells (Jesty et al., 2012; Tallini et al., 2009). A recent study, however, suggested that c-kit-positive cells in the adult mouse heart were capable of regenerating cardiomyocytes after diffuse myocardial damage with isoproterenol (Ellison et al., 2013). The novel aspect in this study was direct evidence with a fate mapping strategy that c-kit-positive cells show in vivo stem cell potential. The authors used a lentiviral-based Cre-Lox strategy to label and track c-kit-expressing cells in the heart. The specificity of the c-kit promoter expression, which is critical to their approach, however, has been questioned (Molkentin and Houser, 2013). Van Berlo and colleagues devised a strategy to genetically label and track c-kit-positive cells and their progeny in the mouse heart. They targeted the *Kit* locus using a Cre recombinase (Kit-Cre) and a tamoxifen-inducible MerCreMer chimeric protein (Kit-MCM), and they bred these mice with a reporter line (Rosa26-eGFP) to permanently label the lineage (van Berlo et al., 2014). Using the Kit-Cre mouse with a constitutively active Cre from the zygote stage, the authors found that c-kit-positive cells contributed to 0.027% of the cardiomyocyte lineage 4 weeks postnatally (80% recombination rate of c-kit-positive cells in the heart), which is much lower than that suggested from previous studies (Hosoda et al., 2009; Kajstura et al., 2010a). Even 4 weeks after cardiac infarction or treatment with isoproterenol, the c-kit-positive cell contribution to the myocyte lineage in the tamoxifen-inducible Kit-MCM mouse was only

0.016% and 0.007%, respectively. Tamoxifen was administered continuously over the whole time period starting two weeks and three weeks prior to injury or only before the ischemic injury with a similar outcome. However, there was a large c-kit lineage contribution to the endothelium that increased after cardiac infarction, in agreement with earlier studies (Sandstedt et al., 2010). The authors further showed that approximately 80% of all recombined EGFP-positive cardiomyocytes resulted from cell fusion. Because tamoxifen was given in one experimental approach strictly before the injury, the possibility of labeling pre-existing cardiomyocytes that then upregulated c-kit expression after injury seems to be unlikely.

Uchida and co-workers performed another fate mapping study based on the tet-cre system to chase sca-1–expressing cells and their descendants in the murine heart (Uchida et al., 2013). The authors showed that sca-1 expression is restricted to the non-myocyte compartment and demonstrated that sca-1-derived cells contribute to a small percentage of all cardiomyocytes at a constant rate during normal aging. Pressure overload, but not ischemic damage, caused a modest increase in the contribution to the pre-existing cardiomyocyte cell pool. The restriction of a subpopulation might have underestimated the extent of cardiomyogenesis, and it is not clear whether sca-1-positive cells divide before differentiation into cardiomyocytes. Because only a minority of sca-1-positive cells in the murine heart resemble CSCs and sca-1 does not exist in humans, further studies are necessary to unveil the exact nature and marker profile of sca-1-positive CSCs. Of note, a subpopulation of sca-1-positive cells, which were negative for the hematopoietic marker CD45 and the endothelial marker CD31, co-expressed the putative CSC marker c-kit (1.6%) (Uchida et al., 2013).

Clinical trials using expanded autologous CSCs (c-kit-positive and cardiosphere-derived cells) have been initiated (Bolli et al., 2011; Malliaras et al., 2013). Patients with post-infarction left ventricular dysfunction received autologous CSCs through their coronary arteries. Preliminary data suggest improved clinical parameters and reduced scar size. However, given the latest lineage tracing studies showing little in vivo contribution of c-kit-positive cells to the cardiomyocyte lineage, the mode of action of the delivered cardiac cells remains unclear. Paracrine effects on myocyte survival and angiogenesis and a direct contribution to the endothelial lineage should be considered, similar to what has been proposed, as a mode of action in the bone marrow mononuclear cell infusion trials (Yoon et al., 2010).

Mature cardiomyocytes as a source for the adult generation of cardiomyocytes

Apart from CSC-derived cardiomyocytes, self-duplication of pre-existing cardiomyocytes has been reported. Adult zebrafish regenerate myocardium after amputation of the apical portion of the heart (Poss et al., 2002). Using a tamoxifen-induced Cre-Lox system under the control of the mature cardiomyocyte-specific cmlc2-promoter, pre-existing cardiomyocytes can be labeled before amputation and chased thereafter. The proportion of labeled cardiomyocytes (EGFP-positive) was unchanged in the regenerated myocardium, indicating that the source was mature cardiomyocytes (Kikuchi et al., 2010). Similarly, a generalized injury (depletion of 60% of the cardiomyocytes in the heart by a suicide gene approach) created a heart failure phenotype, which completely recovered by replication of pre-existing cardiomyocytes within 30 days (Wang et al., 2011). The latter finding would indicate a widespread regenerative capacity within the myocardium of zebrafish. The regenerative capacity of another species, the newt, which has a general high regenerative competence within its adult individuals, has been studied at the single-cell level. Culturing isolated cardiomyocytes revealed that only 1/3 of the cardiomyocytes enter the cell cycle and mitosis, suggesting that even in a highly regenerative species, such as the newt, the myocardium is heterogeneous (Bettencourt-Dias et al., 2003).

Similar to what has been shown in lower vertebrates, the neonatal mouse heart can regenerate myocardium to a substantial extent. Under physiological conditions, cardiomyocyte proliferation decreases to adult levels within the first two weeks, while mouse myocyte become binucleated (Soonpaa et al., 1996; Walsh et al., 2010). Porrello and colleagues showed that an apical amputation of the myocardium at postnatal day one could result in complete regrowth without scar formation within 21 days, although the degree of regeneration has been questioned (Andersen et al., 2014). Regrowth is restricted to the first postnatal days; by postnatal day 7, the hearts fail to regenerate the apex. This process was investigated using an inducible recombination technique similar to that performed in zebrafish using the inducible expression of LacZ in cardiomyocyte (myosin heavy chain isoform alpha expressing cells) (Porrello et al., 2011). Even if the recombination by induction was incomplete (only approximately 60% of the cardiomyocytes was LacZ-positive), the proportion was constant in the apex after injury, suggesting that the source of regenerating cardiomyocyte after injury was mainly pre-existing cardiomyocytes. A similar regenerative response was seen in response to ischemic injury performed at postnatal day 2 (Porrello et al., 2013).

The cell cycle arrest seen postnatally might be explained by an increase in oxygenation compared with the fetal state (Puente et al., 2014). Puente et al. showed that the level of reactive oxygen species (ROS) increases postnatally along with oxidative DNA damage and the DNA damage response (DDR), whereas a reduction of oxygen levels and an inhibition of the DDR response extends the proliferative window in the postnatal heart.

Importantly, mechanisms that govern cardiomyocyte renewal during the neonatal period might be applied to unravel adult myocardial regeneration. For instance, the micro-RNA 15 family and Yes-associated protein (Yap), a transcriptional co-factor in the Hippo signaling pathway, modulate cardiomyocyte renewal not only in neonatal cardiomyocytes but also in adult mouse hearts (Porrello et al., 2013; Xin et al., 2013). Moreover, the homeodomain transcription factor Meis1 regulates cardiomyocyte cell cycle arrest (Mahmoud et al., 2013).

The previously mentioned transgenic model for chasing pre-existing myocytes (EGFP-positive) was also used in adult mice to determine the source of newly born myocytes. The infarcted myocardium was partly replaced with untagged cardiomyocytes (EGFP-negative), suggesting that the new

cardiomyocytes were derived from a more immature source (Hsieh et al., 2007; Loffredo et al., 2011). However, the same research group reported contradictory findings within the same model in studies that were extended with multi-isotope imaging mass spectrometry (MIMS). MIMS allows for the detection of multiple isotopes on ultrathin sections. This technique made it possible to administer ^{15}N thymidine for several months to mice without getting the toxic effects of the commonly used thymidine analog BrdU. In this study, almost all ^{15}N thymidine-incorporating cells were EGFP-positive indicating that pre-existing myocyte self-replicated and generated new myocytes after injury and at a low degree in homeostasis (Senyo et al., 2013). The reason for the reported decrease in EGFP-positive myocytes is not completely understood. Either the recombined myocytes might be more prone to undergo apoptosis or CSCs directly differentiate into cardiomyocytes without progressing through the cell cycle, which would exhaust the CSC pool.

If cardiomyocytes are the source of adult regeneration, then the mechanism for cell cycle re-entry is unknown. Would these cells really exhibit an adult phenotype, which includes an extensive contractile apparatus and a cytoplasm filled with mitochondria? Dedifferentiation of myocytes with the disassembly of the cells' sarcomeric structure, detachment from one another and the expression of cell-cycle regulators has been described in zebrafish (Kikuchi et al., 2010). Disassembled sarcomeres in regions with high proliferative activity have also been reported in injured neonatal hearts (Porrello et al., 2013). Architectural changes in myocytes seem to be required to re-enter the cell cycle and undergo cytokinesis. In injured adult myocardium, the so-called remodeling is connected to the re-expression of genes from fetal and embryonic stages along with myocyte dedifferentiation. These processes may be important to cope with hypoxia and overload-related cell death [for review (Szibor et al., 2013)]. However, why the adult dedifferentiated cardiomyocyte does not undergo cytokinesis to the same magnitude as the neonatal heart is not understood. Molecular profiling strategies comparing the two similar but different age-related stages of myocyte dedifferentiation might provide a more comprehensive understanding of the mechanisms of cycling myocytes.

Future hopes and directions

Efficient myocardial regeneration in humans is the ultimate goal in cardioregenerative medicine. Lower vertebrates, such as zebrafish with their capability to substantially renew the adult myocardium, are important model organisms for the basic understanding of cardiac regeneration. The exciting question why higher vertebrates lose most of their ability to regenerate their hearts after an injury might help us to devise new strategies to trigger the existing regenerative capacity in adult hearts.

Thus, the endogenous generation of cardiomyocytes holds promise because generated *bona fide* cardiomyocytes appear to be well-integrated and (at least in regenerative, lower vertebrate models) have been demonstrated to significantly, not only statistically but also biologically, improve cardiac function after injury.

Additionally, it would be highly desirable to manipulate CSCs to improve their survival and direct them efficiently to the myocyte lineage. A recently developed technology to deliver mRNA in vivo shows promise. Modified RNA (modRNA) encoding human vascular endothelial growth factor-A (VEGF-A) improves heart function and enhances the long-term survival of the murine recipients. These improvements were at least partly mediated by an expansion of epicardial Wt1-positive CSCs and their promotion toward the endothelial and cardiomyocyte lineage (Zangi et al., 2013).

Most studies on adult heart regeneration have been conducted in rodents with hearts that are several hundred-fold smaller than the human equivalents. Furthermore, the expression profile of CSCs might be different in rodents compared with humans. Sca1, for example, is not conserved throughout species and does not exist in humans. The .therapeutic efficiency of CSCs, stem cell-derived cardiomyocytes or small molecules delivered to the myocardium can only be assessed in a clinical-relevant model of cardiac disease. Animal models that better resemble the human situation are therefore greatly needed to translate the knowledge we have obtained in lower vertebrates and rodents to the clinic. Accordingly, in a recent study conducted in non-human primates, Chong and colleagues showed that a graft of human embryonic stem cell-derived cardiomyocytes remuscularized the infarcted macaque heart and electrically coupled to the host myocardium (Chong et al., 2014).

Today, site-specific nucleases such as TALEN and CRISPR/Cas make it possible to introduce custom modifications into genomic DNA (genome editing) [for review see (Gaj et al., 2013)]. This technology allows, for the first time, the generation of transgenic animals without having to establish stable ES cell lines. Recently, a Cre-inducible EGFP reporter pig line was generated using TALEN-mediated genomic editing of the ROSA26 locus (Li et al., 2014). Swine has traditionally served as a model animal for cardiac surgeons because their heart physiology closely resembles the human. Therefore, genome editing technology has the potential to facilitate translational studies to delineate the regulation of myocardial regeneration and thereby facilitate the stimulation of "regenerative" pathways to treat cardiac diseases.

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